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Potential Role of Nitric Oxide in the Pathophysiology of Experimental Bacterial Meningitis in Rats

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We have investigated the possible role of nitric oxide (NO) in the pathophysiology of bacterial meningitis (BM) by using the rat model of experimental BM. The nitrite concentration in cerebrospinal fluid (CSF) was used as a measure of NO production in vivo since NO rapidly degrades to nitrite and nitrate. Rats were inoculated intracisternally with live bacteria (5×10^6 CFU of *Haemophilus influenzae* type b strain DL42 or $Rd^-/b^+/O2$), with bacterial endotoxin (20 ng of DL42 lipooligosaccharide [LOS] or 200 ng of *Escherichia coli* lipopolysaccharide), or with a saline control vehicle. CSF samples were collected preinoculation and at the time of maximal alteration in blood-brain barrier permeability (BBBP). CSF [nitrite] was quantified by measuring A_{550} after addition of the Greiss reagent and comparison to a standard curve of sodium nitrite. Rats inoculated with either DL42, $Rd^-/b^+/O2$, LOS, or lipopolysaccharide demonstrated a significantly elevated mean peak CSF [nitrite] (8.34, 15.62, 10.75, and 10.44 mM, respectively) versus the concentration prior to treatment and/or those in saline-treated animals (5.29 and 5.33 mM, respectively; $P < 0.05$ for each comparison). We then determined if there was a correlation between CSF [nitrite] and percent BBBP (%BBBP) at various time points postinoculation with $Rd^-/b^+/O2$. %BBBP was defined as the concentration of systemically administered ^{125}I-labeled bovine serum albumin in the CSF divided by the level of ^{125}I-labeled bovine serum albumin in serum multiplied by 100. The mean %BBBP increased in tandem with the mean CSF [nitrite] ($R = 0.84$, $P = 0.018$), which peaked at 18 h in the absence of a change in the serum [nitrite]. Systemic administration of the NO synthase inhibitor *N*-nitro-L-arginine methyl ester demonstrated a significant reduction of mean CSF nitrite production (0.95 versus 6.0 mM in controls; $P = 0.02$) when administered intravenously to animals which had been inoculated intracisternally with 20 ng of LOS. Suppression of mean leukocyte pleocytosis (3,117 versus 11,590 leukocytes per mm^3 in control LOS-challenged rats; $P = 0.03$) and mean alterations of BBBP (2.11 versus 6.49% in control LOS-challenged rats; $P = 0.009$) was observed concomitantly with decreased CSF [nitrite]. These results support the hypothesis that NO contributes to increased %BBBP in experimental BM.

Bacterial meningitis (BM) is an inflammatory disease of the central nervous system (CNS) which occurs when bacteria gain entry to the subarachnoid space. In one model of the pathophysiologic alterations resulting from BM (47), bacteria release cell surface components into the cerebrospinal fluid (CSF) (e.g., lipopolysaccharide [LPS]) which trigger resident CNS cells to produce inflammatory cytokines, specifically, interleukin-1 and tumor necrosis factor (48, 51, 62). Following these early events, these cytokines induce a number of cellular processes that culminate in the chemoattraction of neutrophils, adherence of neutrophils to cerebromicrovascular endothelium, and subsequent diapedesis of the neutrophils into the CSF (40, 57). Once the neutrophils have entered the CSF, exposure to various stimuli induces release of a number of products, including reactive oxygen species such as superoxide and hydroxyl radicals that may contribute to the neurologic damage seen during BM as the disease progresses.

Another reactive oxygen species that may be involved in the pathophysiology of BM is nitric oxide (NO). NO has been shown to be produced by a number of mammalian cell types, including endothelial cells, phagocytes, and resident CNS cells (17, 25, 55), and is produced by at least three different synthases (56). The molecule plays a variety of physiological roles, acting as a vasodilator (6, 37, 59), an antimicrobial agent (2, 3, 14, 24, 32), at least in some systems, and a neurotransmitter (5, 9, 38). Synthesis of NO can be induced by a variety of stimuli, including bradykinin, gamma interferon, tumor necrosis factor, and LPS (7, 17, 42).

Within the last 2 years, four observations have suggested that NO plays a role in the pathophysiology of BM. First, in an experimental rat model of pneumococcal BM, treatment with an NO synthase inhibitor attenuated a number of early acute events associated with BM, such as increases in regional blood flow, intracranial pressure, brain water content, and leukocyte (WBC) pleocytosis (46). Second, coincubation of rat astrocytes in primary culture with pneumococci stimulated NO production and was inhibited by NO synthase inhibitors (4). Third, Kornelisse and colleagues documented significantly increased [nitrite] (a breakdown product of NO synthesis) in the CSF of a small number of patients with meningococcal meningitis. This increased CSF [nitrite] occurred in the absence of an increase in [nitrite] in serum (27). Finally, increased CSF [nitrite] in seven patients with meningitis (three viral, four bacterial) was also observed by Milstein et al. (35). We have confirmed increased CSF [nitrite] in a limited number of BM patients (unpublished data).

We hypothesize that NO is involved in the pathophysiology

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of BM and may play a role in the altered blood-brain barrier (BBB) permeability (BBBP), cerebral blood flow, brain edema, and other potential consequences of subarachnoid space inflammation. Specifically, the purposes of this study were (i) to compare the CSF [nitrite] of rats inoculated with live bacteria or with bacterial endotoxin with the CSF [nitrite] of noninoculated rats or rats inoculated with saline, (ii) to correlate the increase in %BBBP in rats inoculated with live bacteria with CSF [nitrite], and (iii) to assess the BBBP response in experimental BM after treatment with an NO synthase inhibitor.

MATERIALS AND METHODS

Bacteria. *Haemophilus influenzae* DL42 and $Rd^-/b^+/O2$ were kept at −70°C in skim milk. The bacteria were plated onto chocolate agar (Becton Dickinson, Cockeysville, Md.) and incubated overnight at 37°C in 5% CO_2. Suspensions of the bacteria were made in phosphate-buffered saline at a concentration of 10^8 CFU/ml. Strain DL42 was originally provided by Eric Hansen, University of Texas Southwestern Medical Center, and is a clinical isolate from an invasive infection and widely used in work in this laboratory (35, 47, 48, 62). Strain $Rd^-/b^+/O2$ is a transformant of the Rd strain made by using donor DNA from type b strain Eagan (19) and is fully virulent in the rat model (30).

An aliquot of the bacterial solution was incubated at 37°C for 18 h and then centrifuged at 3,000 × *g* for 15 min to pellet the bacteria. The supernatant was then assayed for nitrite as described below.

Endotoxin. *H. influenzae* DL42 lipooligosaccharide (LOS) was supplied by Eric Hansen after extraction and purification as described elsewhere (62) and stored at −70°C. *Escherichia coli* 026:B6 LPS (Sigma, St. Louis, Mo.) was resuspended in normal saline. Aliquots for inoculation were sonicated for 3 min with a W140D Sonifier (Ultrasonics-Heat Systems, Inc., Plainview, N.Y.) and then diluted in normal saline to achieve the desired concentration. Doses used were based on the results of previous studies which demonstrated that maximal WBC concentrations in CSF and percent BBBP to systemically administered radiolabeled albumin (%BBBP) were elicited by inoculation of 20 ng of *H. influenzae* LOS (48, 62) and by preliminary experiments in this laboratory demonstrating the same obtained with 200 ng of *E. coli* LPS (data not shown).

Meningitis model. Adult Wistar rats (approximately 200 g) were anesthetized with intramuscular injections of ketamine (75 mg/kg) and xylazine (5 mg/kg) (Barber Veterinary Supply, Lynchburg, Va.). In experiments in which the CSF [nitrite] in response to endotoxin or bacteria was examined, LOS (20 ng), LPS (200 ng), and *H. influenzae* strains (5×10^6 CFU) were inoculated via percutaneous puncture of the cisterna magna after withdrawal of 50 μl of CSF. CSF was then sampled at either 4 h postinoculation with endotoxin or 18 h postinoculation with bacteria, and CSF [nitrite], alterations in BBBP, and WBC counts were determined. WBC concentrations in CSF were determined by standard hemacytometer methods. These time periods were chosen because they reflect times of maximal alterations in BBBP previously observed in this model.

In experiments in which an NO synthase inhibitor was administered, LOS (20 ng) was inoculated as described above. The NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 5 mg/kg; Sigma) (53) was administered intravenously at 0, 1, 2, and 3 h postinoculation via a 25-gauge catheter (Critikon, Tampa, Fla.) placed in a tail vein. CSF was then sampled at 4 h postinoculation, and CSF [nitrite], alterations in BBBP, and WBC counts were determined.

Nitrite assay. To deproteinize serum, 25 μl of serum was diluted with 130 μl of phosphate-buffered saline. The diluted serum was mixed with 25 μl of 0.5 M zinc acetate (Sigma), and the sample was sonicated for 10 min. The sample was then assayed for nitrite as described above. For assessment of [nitrite], 25 μl of a CSF or deproteinized serum sample was added to an equal volume of a 2.4 M ammonium formate–1.0 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer. This mixture was vortexed and incubated for 1 h at 37°C, after which 37 μl was mixed with an equal volume of an aqueous solution of 0.5% sulfanilamide (Sigma)–0.01% naphthylethylenediamine (Sigma) and incubated at room temperature for 15 min. The A_{550} was read in a Titertek Multiskan Plus spectrophotometer (Flow Laboratories, McLean, Va.) and compared with a sodium nitrite standard curve in Krebs-Hensleit buffer (61).

Serum nitrite was assayed by reduction of the nitrate in the samples to nitrite. A sample (50 μl) or a sodium nitrate standard (50 μl) was added to 150 μl of phosphate-buffered saline (pH 7.5). NADPH-dependent nitrate reductase (Sigma) was added, and the sample was vortexed, incubated for 2 h at room temperature, mixed (37 μl) with an equal volume of Greiss reagent, and incubated at room temperature for 15 min, after which the A_{550} was read as described above.

BBBP assay. Anesthetized rats were given an intracardiac injection of ^{125}I-labeled bovine serum albumin (ICN Radiochemicals, Irvine, Calif.) concomitantly with intracisternal inoculation of $Rd^-/b^+/O2$ (5×10^6 CFU). CSF and blood samples were taken at frequent intervals over a 24-h period (two samples were taken per time period). For assessment of %BBBP, 25 ml of each sample of CSF and blood was read simultaneously in a gamma counter. After subtraction of background radioactivity, %BBBP was calculated by the following formula: %BBBP = (counts per minute in CSF/counts per minute in blood) × 100.

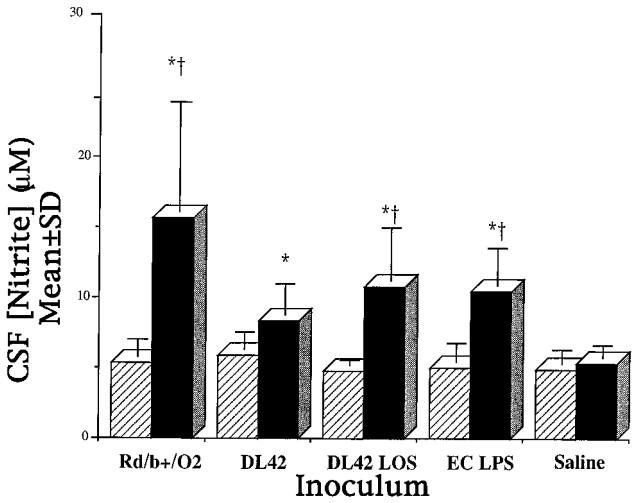

FIG. 1. CSF [nitrite] in rats after intracisternal challenge with LOS (20 ng), LPS (200 ng), *H. influenzae* $Rd^-/b^+/O2$ or DL42 (5×10^6 CFU), or saline. CSF was sampled at either 4 h postinoculation with endotoxin or 18 h postinoculation with bacteria, and CSF [nitrite], alterations in BBBP, and WBC counts were determined. ▨, before inoculation; ■, postinoculation; *, $P < 0.05$ compared with inoculation with saline; †, $P < 0.05$ compared with preinoculation value. EC, *E. coli.*

Statistics. All statistical tests were performed with Instat biostatistical software (Graphpad, San Diego, Calif.) to compare postinoculation samples from rats inoculated with bacteria or endotoxin with either the corresponding preinoculation samples or with postinoculation samples from rats inoculated with saline. The statistical test used was the Student *t* test, except in the %BBBP and CSF [nitrite] time course experiments and for linear correlation determination, for which the analysis of variance test was used to generate the *P* value.

RESULTS

We assayed for the presence of nitrite as an indicator of NO production in CSF and blood. Figure 1 shows the mean CSF [nitrite] postinoculation with live bacteria, bacterial endotoxin, or saline. Rats inoculated with either live bacteria or endotoxin exhibited significantly higher CSF [nitrite] than did uninoculated rats or rats inoculated with saline. For example, the mean CSF [nitrite] peaks were 8.34 and 10.44 mM for animals inoculated with DL42 ($P = 0.05$) and DL42 LOS ($P = 0.03$), respectively, compared with 5.33 mM for animals inoculated with saline. Saline inoculation alone did not alter CSF [nitrite] (5.29 versus 5.33 mM; $P = 0.53$). All values for rats that received live bacteria, LPS, or LOS were significantly greater than those for saline controls ($P < 0.05$). The animals treated with $Rd^-/b^+/O2$ demonstrated the greatest increase in CSF [nitrite], with a mean value of >10 mM over baseline concentrations. To answer the question of whether the bacteria could be the source of nitrite, the bacterial supernatants were assayed and were shown to contain no detectable nitrite (data not shown).

The potential correlation of increased %BBBP and increased CSF [nitrite] was examined in rats infected with $Rd^-/b^+/O2$ as shown in Fig. 2. CSF [nitrite] and %BBBP both began to increase at approximately 4 h postinoculation and reached maximal levels 18 h later. The coefficient of correlation between increased %BBBP and increased CSF [nitrite] was 0.86 ($P = 0.018$). However, there was no linear correlation between increased CSF WBC levels and increased CSF [nitrite] ($r = 0.0$, $P = 0.74$; data not shown).

Nitrite levels in serum were assessed to address the possi-

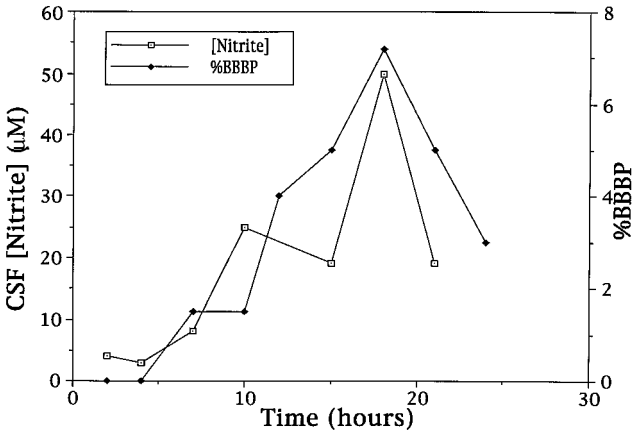

FIG. 2. Correlation of CSF [nitrite] and alterations in BBBP in the rat model of experimental BM. Anesthetized adult Wistar rats were given an intracardiac injection of ^{125}I-labeled bovine serum albumin (ICN Radiochemicals) concomitantly with intracisternal inoculation of $Rd^-/b^+/O2$ (5×10^6 CFU). CSF and blood samples were taken over a 24-h period.

bility that the serum was the actual source of the nitrite in CSF. In addition to nitrite determinations, serum was assayed for nitrate to explore the chance that serum nitrite was completely converted to nitrate. When nitrite and nitrate concentrations in serum were assessed over the same time period, no increase over the background was detected (data not shown); this indicated that the increased [nitrite] in the CSF was produced locally.

Table 1 demonstrates the effect of the NO inhibitor L-NAME on WBC pleocytosis and alterations of BBBP in rats inoculated with LOS. In rats treated with L-NAME, both the mean CSF WBC counts (3,117 WBCs/mm^3) and the mean %BBBP (2.11%) were significantly reduced compared with those of rats which received no NO synthase inhibitor (11,590 WBCs/mm^3 [$P = 0.035$] and 6.49% [$P = 0.009$]). Infusion with L-NAME also demonstrated significant suppression of the mean CSF [nitrite] in comparison with controls (0.95 versus 6.0 mM, respectively; $P = 0.02$). In addition, L-NAME demonstrated a significant reduction of the mean CSF [nitrite] below baseline levels (as seen in Fig. 1).

DISCUSSION

In the experiments presented here, we have begun to examine the potential role of NO in the pathophysiology of experimental BM. We have demonstrated that the mean CSF [nitrite] of rats inoculated with live bacteria (*H. influenzae* DL42 and $Rd^-/b^+/O2$) or endotoxin (*H. influenzae* LOS and *E. coli* LPS) was significantly higher than the mean CSF [nitrite] of noninoculated rats or of rats inoculated with saline. In animals inoculated with $Rd^-/b^+/O2$, a correlation between increased CSF [nitrite] and increased BBBP with maximal BBBP and CSF [nitrite] reached at 18 h was demonstrated. Serum [nitrite] over the same time periods failed to exhibit any detectable increase over background levels. Infusion of an NO synthase inhibitor decreased both the mean CSF WBC count and the mean %BBBP associated with a decrease in CSF [nitrite] compared with those in rats after an LOS challenge. The suppression of CSF nitrite production by L-NAME below the baseline level may be due to inhibition of constitutive NO synthases, as well as inducible NO synthases.

While there is no linear correlation between CSF nitrite production and CSF WBC pleocytosis, the ability of the NO synthase inhibitor L-NAME to reduce WBC concentrations in CSF indicates that there is some association between NO production and WBC entry into the subarachnoid space. This may be explained by the ability of WBCs to migrate across the endothelial barrier. It is possible that NO contributes to the general inflammatory response during BM, thus promoting production of chemotactic factors and WBC-endothelial cell adhesion molecules which facilitate entry of WBCs into the subarachnoid space.

NO has been implicated in the pathophysiology of inflammation in a number of organ systems. Administration of NO synthase inhibitors to animals with a variety of experimental diseases, such as adjuvant arthritis, immune complex-induced pulmonary vascular injury, and chronic ileitis, attenuated the inflammation while administration of L-arginine (the NO precursor molecule) enhanced the disease states (20, 21, 34, 41, 61). Increased NO or nitrite production was observed in patients suffering from ulcerative colitis, from rheumatoid arthritis, or from osteoarthritis in addition to the experimental model data (8, 33). The above experimental and clinical observations are in strong agreement with the observations presented in this report, as well as that of Kornelisse and colleagues demonstrating increased CSF [nitrite] in pediatric patients with meningococcal meningitis (27) and the observations of Pfister and colleagues that NO synthase inhibitors attenuated the early altered pathophysiology of experimental acute BM in a rat model (46).

The mechanism by which NO may contribute to the pathophysiology of BM is not understood. NO produced by phagocytes has been shown to be either cytocidal or cytostatic for a variety of cells, including tumor cells (14, 18, 64), protozoa (2, 13, 14, 24), bacteria (1, 32), and fungi (3), in various experimental in vitro systems. Within the target cells, NO disrupts various enzyme systems associated with mitochondrial respiration, DNA replication, and the citric acid cycle (7, 10–12, 18). The enzyme inactivation is accomplished by NO chelation of iron cofactors necessary for the function of these enzymes (16, 29, 39, 45, 58). A plausible scenario in which NO inactivates these iron-containing enzyme systems in the microvascular endothelial cells that constitute a major site of the BBB, thereby causing cellular destruction or alteration and loss of integrity of the BBB, may be postulated.

In addition to its enzyme inactivation capability, NO can also react with the heme group of guanylate cyclase, increasing production of cyclic GMP (31). This increased cyclic GMP can trigger smooth muscle relaxation, leading to vasodilatation of the microvascular network (36, 52, 60). The role of this vasodilatation in the CNS and its consequence for the integrity of the BBB tight junctions and the pathophysiology of BM are unknown.

NO has also been shown to initiate the production of other strong oxidants which may contribute to cellular destruction and alterations in CNS homeostasis. One such strong oxidant

TABLE 1. Effect of the NO inhibitor L-NAME on WBC pleocytosis, alterations of BBBP, and CSF [nitrite] in rats inoculated with LOS

Treatment	Mean no. of WBCs/mm^3 in CSF ± SD	Mean %BBBP ± SD	Mean CSF [nitrite] ± SD
Without L-NAME	11,590 ± 8,087	6.49 ± 4.5	6.0 ± 5.1
After L-NAME	3,711 ± 2,728[a]	2.1 ± 1.6[b]	0.95 ± 2.4[c]

[a] $P = 0.03$ compared with LOS-meningitis control.
[b] $P = 0.01$ compared with LOS-meningitis control.
[c] $P = 0.02$ compared with LOS-meningitis control.

is peroxynitrite, which has been shown to be produced through a reaction of NO with superoxide anion (22–24, 26, 36, 52, 60). Peroxynitrite causes oxidation of sulfhydryl groups in single amino acids and polypeptides (49), nitration of tyrosine (22), and lipid peroxidation (50). These peroxynitrite-induced modifications could interfere with critical metabolic activities of membrane and cytoplasmic moieties by destroying the active sites on these molecules. In fact, it has been demonstrated that peroxidation of membrane lipids is associated with cellular dysfunction (15, 28, 54). Peroxynitrite may well be more injurious than NO itself during the disease process.

While the source of nitrite in our BM model is unknown, there are at least three separate mammalian cells (in addition to neurons) which exhibit the ability to produce nitrite in vitro at levels sufficient to explain our in vivo observations and may function as the source(s) of NO. In vitro experiments with primary rat astrocyte cultures have demonstrated the ability of these cells, resident CNS cells of obvious importance (42), to produce an inhibitable nitrite response when incubated with heat-inactivated, unencapsulated pneumococci (4). The second possible source of CSF nitrite is the microvascular endothelium which constitutes the BBB. A number of studies have shown that vascular endothelial cells possess an inducible NO synthase that produces NO in response to mediators of inflammation (4, 43–45, 53, 54, 56). It is not known if L-NAME crosses the BBB; however, if it were demonstrated that L-NAME does not cross the BBB, then the most likely candidate for the source of NO in our model would be the cerebromicrovascular endothelium. Finally, a third possible source of CSF nitrite may be the neutrophils that cross into the CSF during BM (32, 55, 63). As shown by in vitro experiments, both human and rat neutrophils have demonstrated the ability to synthesize relatively high levels of nitrite when activated by various stimuli. Further studies are essential to investigate the sources of the elevated [nitrite] we have observed.

In conclusion, we have demonstrated an increase in [nitrite] in the CSF of rats with experimental BM and found that this nitrite production appears to correlate with elevated BBBP from 2 to 24 h postinoculation with live organisms. Also, we have demonstrated that when an NO synthase inhibitor was employed, decreased CSF [nitrite] corresponded to decreased WBC counts in the CSF and decreased %BBBP. The source of the CSF nitrite is unknown but may be astrocytes, neutrophils, or endothelial cells, alone or in combination, in the CNS during the experimental infection. The mechanism whereby NO exerts its influence on the pathophysiology of BM may be by initiation of vasodilatation of the microvascular endothelium or alteration of these endothelial cells via inhibition of the normal function of various essential metabolic and/or structural molecules. In light of these observations, it is feasible that agents which specifically inhibit NO synthesis in the CNS during BM may be used as an adjunct therapy to potentially reduce the morbidity and mortality associated with this disease.

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